NJC

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/njc

New Journal of Chemistry

COMMUNICATION

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Regulation of collagen expression using nanoparticle mediated inhibition of TGF-β activation

Binbin Li,[ab] Tristan D Clemons,[c] Vipul Agarwal,[c] Jessica Kretzmann,[c] Michael Bradshaw,[c] Priyanka Toshniwal,[c] Nicole M Smith,[c] Shipu Li,[ab] Mark Fear,[de] Fiona M Wood,[de] K. Swaminathan Iyer[c]*

PXS64 is a stable mannose-6-phosphate (M6P) analogue which has been shown to be an effective anti-fibrotic agent by inhibiting the activation of latent TGF-β1. However PXS64 is insoluble in physiological conditions. Herein, we report a multifunctional poly(glycidyl methacrylate) (PGMA) polymeric nanoparticle system for intracellular delivery of PXS64 in human primary scar cells. We demonstrate the efficacy of this anti-fibrotic platform by monitoring the expression of collagen production using an *in vitro* scar model.

Skin injuries and scarring represent a huge global burden with billions of dollars spent annually on the treatment of scars.[1] In humans, wound healing occurs not through a regenerative process that restores normal tissue architecture but through a reparative process that results in scar formation.[2] As a result of significant advances in critical care reducing mortality, the development of scarring following severe injury remains one of the significant barriers to function and the most visible stigma of injury. The long-term sequelae of severe scarring includes pruritus, physical pain, restriction of mobility and altered skin tension.[3, 4] Coupled with the common psychological effects such as depression, sleep disorders and poor body image perception, scarring has a long-term impact on the quality of life of patients after significant injury.[4]

Scar is functionally and aesthetically inferior to normal skin.[5] It occurs as a result of the increased production of extracellular matrix (ECM) that occurs after injury. This increased ECM production is driven initially by inflammation, and is essential to reduce the risk of infection and promote rapid repair. Once the altered matrix is established, despite prolonged periods of remodelling, normal architecture is never restored.[2, 5] Transforming growth factor beta (TGFβ) is a key regulator of matrix deposition after injury, with excessive activation linked to hypertrophic and poor scars.[6, 7] TGFβ has a suppressive effect on the inflammatory response and promotes the formation of granulation tissue. TGFβ stimulates fibroblasts to produce increased levels of ECM molecules including collagen, fibronectin and matrix associated glycosaminoglycan's (GAGs).[8] However TGFβ in the matrix is stored in a latent inactive form.[6] Activation requires separation of the pro-domain from the active growth factor domain. This can be stimulated through mannose-6-phosphate (M6P) groups found on the latency-associated peptide binding to the M6P receptor. Due to the importance of this M6P interaction for TGFβ activation, Mannose-6-phosphate has long been pursued for its anti-fibrotic potential. Studies have shown that exogenous M6P can significantly down regulate TGFβ induced collagen production in a range of *in vitro* and *in vivo* models making it an exciting therapeutic prospect.[9, 10] As a result of this, M6P was progressed through phase II clinical trials by Renovo, under the trade name of Juvidex® for the treatment of split thickness skin grafts.[11] Intradermal delivery of Juvidex® was shown to significantly accelerate the wound healing process however produced no significant effect on the extent of scar formation observed.[12] This is most likely at least in part due to the difficulty in subjectively assessing scars but is also likely to be a result of the poor biological stability of M6P. M6P is highly vulnerable to phosphatase degradation, a drawback considered to be a major limitation for M6P in clinical applications.[13] Hence the development of M6P analogues with improved stability as well as suitable delivery platforms to further enhance their stability in a biological setting could be advantageous. Herein, we have developed a nanoparticle delivery platform suitable for the delivery of a hydrophobic M6P analogue, PXS64, and tested the effects of this anti-scarring therapeutic in an *in vitro* model of scarring.

This study made use of PXS64 loaded polymeric nanoparticles synthesised from poly(glycidyl methacrylate) (PGMA) cores and containing a cationic polyetheleneimine (PEI) surface coating (Figure 1A). PXS64 is a hydrophobic pro-drug designed by Pharmaxis Pty. Ltd., developed to be resistant to phosphatase degradation, which upon cellular internalisation undergoes chemical

[a] State Key Laboratory of Advanced Technology for Materials Synthesis and Processing, Wuhan University of Technology, Wuhan 430070, PR China
[b] Biomedical Materials and Engineering Research Center of Hubei Province, Wuhan 430070, PR China.
[c] School of Chemistry and Biochemistry, The University of Western Australia, Crawley WA 6009, Australia. E-mail: swaminatha.iyer@uwa.edu.au
[d] Fiona Wood Foundation and Burn Injury Research Unit, M318, The University of Western Australia, 35 Stirling Highway, Crawley, WA, 6009 (Australia).
[e] Burns Service of Western Australia, Royal Perth and Princess Margaret Hospitals, WA, 6000, Australia.

† Electronic Supplementary Information (ESI) available: Complete experimental details and supplementary Figures S1 – S4. See DOI: 10.1039/x0xx00000x

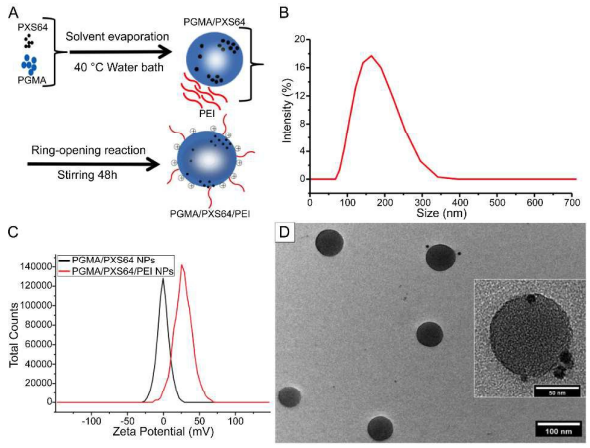


Figure 1. Schematic of nanoparticle synthesis and characterisation. (A) Schematic of the PGMA/PXS64/PEI nanoparticle synthesis process. (B) PGMA/PXS64/PEI nanoparticle size distribution as assessed by DLS. (C) Zeta potential of the PGMA/PXS64 nanoparticles before PEI modification (black), and with PEI modification (red). (D) TEM image of PGMA/PXS64/PEI nanoparticles, scale 100 nm and inset 50 nm.

modification by endogenous esterases to yield an active M6P analogue (see supporting information, Figure S1).[14-16] The attachment of cationic polymers to the surface of polymeric nanoparticles has long been established as an integral modification to enhance nanoparticle interaction with cells and in turn cellular uptake and was therefore hypothesised to be advantageous for the purpose of delivering the anti-fibrotic compound.[17, 18] Furthermore, PEI functionalised nanoparticles have been demonstrated to be non-toxic and stable from aggregation in both *in vitro* and *in vivo* studies.[19, 20] The use of a PGMA core allowed for the covalent attachment of the PEI chains to the nanoparticle surface as well as the covalent attachment of rhodamine B to the nanoparticles for fluorescent tagging in cellular uptake studies. Short chain PEI was used for nanoparticle surface modification to avoid PEI cytotoxicity, which has been shown to increase with increasing molecular weight.[21] The PGMA/PXS64/PEI nanoparticles used in this study had an average hydrodynamic diameter of 154 nm (PDI 0.121) as determined by dynamic light scattering (Figure 1B) and a final surface charge of 27.5 ± 0.5 mV following the PEI conjugation to the PGMA/PXS64 nanoparticle surface (Figure 1C). The nanoparticles were uniform and spherical in nature as confirmed by transmission electron microscopy (TEM, Figure 1D and supporting information Figure S2). Drug loading of the nanoparticles was determined *via* high-pressure liquid chromatography (HPLC) where the drug loading of the nanoparticles was calculated to be 3.06% (w/w).

The toxicity and biocompatibility of the PGMA/PXS64/PEI nanoparticle system was assessed on human primary dermal scar fibroblasts with the MTS assay, a measure of cell proliferation over time.[22] Proliferation was assessed at 24 and 48 h following incubation with the PGMA/PXS64/PEI nanoparticle system up to a maximum concentration of 200 µg/ml. No significant reduction in cell proliferation was evident across both time points and all concentrations (Figure 2, $p<0.05$). Further to this the MTS assay was also performed for comparative PXS64 concentrations (maximum

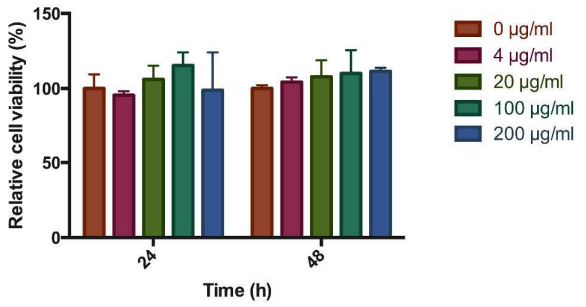


Figure 2. MTS cell viability assessment of human primary dermal scar fibroblasts after treatment with different concentrations of PGMA/PXS64/PEI NPs at both 24 and 48 h following incubation. Data displayed as relative cell viability compared to control for each time point ± standard deviation, significance assessed with an ANOVA followed by a Bonferroni post-hoc test with $p<0.05$.

concentration 20 µM) and PGMA/PEI nanoparticles without drug loading (maximum concentration 400 µg/ml) (See Supporting information, Figure S3). No significant difference in cell viability between controls and both the drug alone and the nanoparticles without drug (PGMA/PEI) treatments was evident at 48 h following incubation.

Targeting of the mannose 6-phosphate/insulin-like growth factor II (M6P/IGFII) receptor has long been a highly attractive therapeutic target due to the role this receptor plays with regards to fibroblast cell signalling during fibrosis.[15, 23] Importantly, it modulates the activity of a variety of extracellular M6P bearing glycoproteins like latent TGFβ precursor, urokinase-type plasminogen activator receptor, glycoprotein D of the herpes virus, granzyme B an essential factor for T cell-mediated apoptosis and proliferin-induced angiogenesis.[24] It has been shown previously that of the M6P/IGFII receptors, as little as 10% are found on the cell surface with the remaining majority localized in the compartments of the cell.[15] This, coupled with a relatively long receptor half-life ($t_{½} \approx$ 20h) makes targeting only the M6P/IGFII receptors on the cell surface likely to be less effective than an approach that can target intracellular receptors.[25] PXS64 is a lipophilic molecule, which upon cellular internalisation, undergoes ester hydrolysis to produce an active M6P analogue. This significantly increases its resistance to phosphatase degradation in comparison to M6P. This coupled with nanoparticles designed for rapid cellular uptake and in turn delivery of the relatively more stable M6P analogue, PXS64, provides a means to overcome the aforementioned issues associated with M6P alone. For this study, firstly it was important to confirm that the nanoparticle construct was able to effectively target and associate with primary human dermal scar fibroblasts. For this the PGMA/PEI nanoparticle construct was slightly modified *via* the covalent attachment of the fluorescent dye, rhodamine B, to the nanoparticles PGMA core. This allowed for nanoparticle tracking by confocal imaging in an *in vitro* setting. Nanoparticles were incubated with primary human dermal scar fibroblasts for 48 hours and strong cellular association was demonstrated with the scar cells using both confocal and fluorescence imaging (Figure 3 and S4 respectively). It was anticipated that this rapid nanoparticle

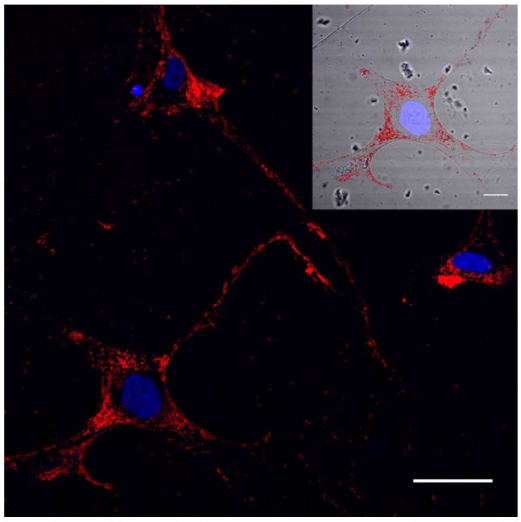


Figure 3. Fluorescent uptake of PGMA/PEI/RhB NPs following 48 h incubation with 10 ug/ml nanoparticles, as assessed by confocal microscopy. Rhodamine labelled nanoparticles (red) and Hoescht (blue) labelled nuclei, inset fluorescent overlay on bright field image. Scale bars: 50 µm with inset 20 µm.

association with fibroblasts would allow for the direct intracellular release of the PXS64 therapeutic cargo.

Finally, the role of PXS64 loaded nanoparticles was investigated in the scar-in-a-jar *in vitro* model of scarring. This model developed by Chen and Raghunath is a highly relevant model of scarring avoiding the long cell culture times (4-6 weeks) required for similar fibroplasia models.[26-28] The scar-in-a-jar model makes use of the stimulating effects of TGFβ, similar to common fibroplasia models, however it is able to accelerate the amount of collagen deposition due to the addition of 'crowding' macromolecules to the cell media to produce a 'scar like' cellular environment.[26] Another advantage of this model is that the collagen deposited can be imaged and assessed in its native state where other models often require destructive pre-solubilisation of the deposited collagen for quantification. Human primary dermal scar fibroblasts were stimulated with TGFβ and induced into a 'scar like' state with the introduction of macromolecular crowding media (see Supporting Information for methods). The cells were treated with either PGMA/PEI nanoparticles (no drug loaded) or drug loaded PGMA/PXS64/PEI nanoparticles and the effect on collagen deposition assessed with immunocytochemistry and fluorescence microscopy. PGMA/PXS64/PEI drug loaded nanoparticles were able to induce a significant reduction in the amount of collagen deposited per cell (approximately 60% reduction) even at a concentration as low as 4 µg/ml, which corresponds to a PXS64 total drug concentration of 0.2 µM (Figure 4A). Free drug PXS64 treatments in the same model found a 70% reduction in collagen expression with a concentration of 1 µM, and complete inhibition of collagen deposition at a concentration of 10 µM (See supporting information, Figure S5). Immunocytochemistry of collagen I clearly demonstrates a reduction in collagen deposition per cell with treatments of PGMA/PXS64/PEI and PXS64 alone both showing significant reductions in the deposited collagen (Figure 4B – 4E). At

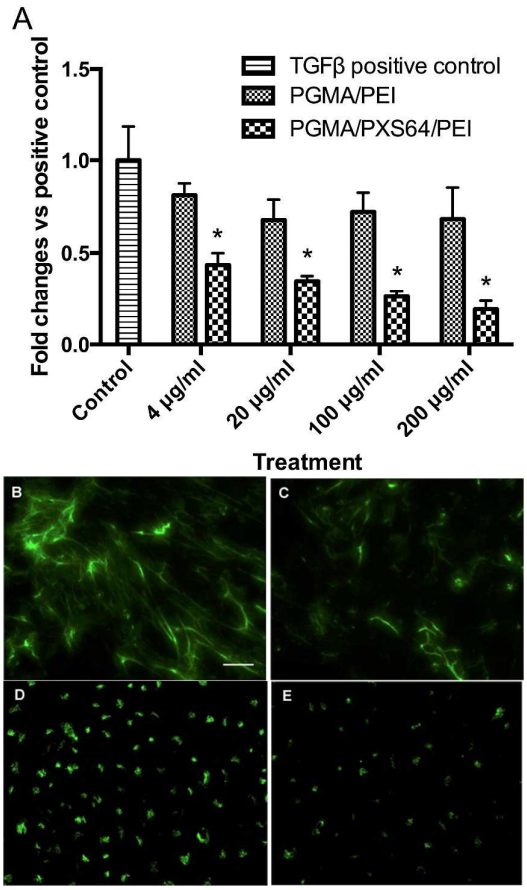


Figure 4. Collagen deposition analysis and morphology observation from scar-in-a-Jar *in vitro* study. A. Collagen deposition per cell (area deposited) treated with different concentrations of PGMA/PXS64/PEI or PGMA/PEI NPs (no drug). Fluorescence microscopy images of the deposited collagen morphology for B. control (no treatment) C. PGMA/PEI NPs (200 µg/ml), no drug, D. PGMA/PXS64/PEI NPs (200 µg/ml), E. PXS64 alone (10 µM). Data displayed as mean ± standard deviation, significance assessed with an ANOVA followed by a Bonferroni post-hoc test with $p<0.05$. Scale bar: 100 µm.

equivalent levels of PXS64 delivered as either free drug or from within the PGMA/PEI nanoparticle delivery vehicle, the level of collagen inhibition was similar. It is anticipated that the lipophilic nature of the PXS64 will result in a sustained release effect from the nanoparticle. This potential effect is difficult to assess in a short term *in vitro* model but likely to have benefits for long-term scar therapies and in an *in vivo* setting.

Herein we have demonstrated the effective loading of a lipophilic M6P analogue in a suitable nanoparticle platform to enhance its cellular uptake for application in the treatment of scarring. The drug-loaded nanoparticle elicited no toxic side effects when assessed in human primary dermal scar fibroblasts and the nanoparticles were proven to associate rapidly with these cells as assessed by confocal microscopy. The drug-loaded nanoparticles reduced the amount of collagen secretion of human primary dermal scar fibroblasts in a recognised *in vitro* scar model. The role of nanoparticle-assisted delivery has the potential to greatly enhance

the application of M6P analogues in scar treatment and the role of PXS64 in the reduction of collagen presented here suggests clinical potential for this delivery mechanism in the use of anti-fibrotic agents.

Experimental

Nanoparticle synthesis and characterisation

All nanoparticles were synthesized making use of an emulsion synthesis protocol described previously[19] and detailed in full in the electronic supplementary information. Dynamic light scattering (DLS) measurements of size and zeta potential of nanoparticle preparations were performed on a Malvern Zetasizer instrument. Samples prepared for transmission electron microscopy (TEM) analysis were prepared by depositing onto carbon-coated grids and imaged at 120kV on a JEOL JEM-2100. High Performance Liquid Chromatography (HPLC) analysis was conducted on a Waters 2695 instrument with a Waters 2489 UV/Vis detector. Samples were run on a C18 column with an acetonitrile/water gradient solvent system (both containing 0.1% trifluoroacetic acid), flow rate of 1ml/min and absorbance detected at a wavelength of 280nm.

Cell Culture

Human primary fibroblast scar cells used herein were cultured in T75 flasks in a humidified atmosphere containing 5% CO_2 at 37 ^{o}C, and maintained in DMEM/F12 medium containing fetal bovine serum (10% v/v) and penicillin/streptomycin (1% v/v). Cell viability measurements were carried out using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS; Promega) following standard protocols. Scar-in-a-jar analysis was conducted following previously published procedures[26] and is outlined in full in the electronic supplementary information.

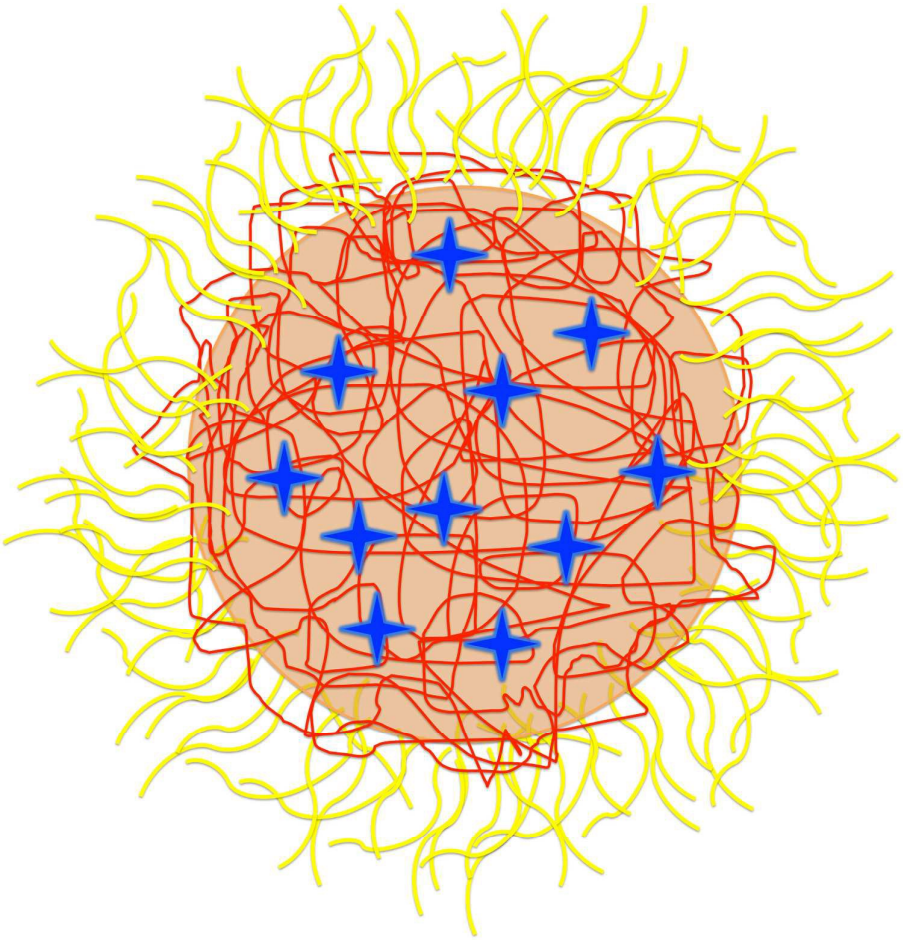
Acknowledgements

The Australian Research Council (ARC), and the National Health & Medical Research Council (NHMRC) of Australia funded this work. The authors acknowledge the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, the University of Western Australia; funded by the University, State and Commonwealth Governments. The authors would like to acknowledge the gracious support from Pharmaxis Pty. Ltd. For providing the PXS64 used in this study. TC is a NHMRC Peter Doherty – Australian Biomedical Fellow, KSI is an ARC Future Fellow and MF is supported by Chevron Australia. The Fiona Wood Foundation graciously provided the cells for this work.

Notes and references

1. C. K. Sen, G. M. Gordillo, S. Roy, R. Kirsner, L. Lambert, T. K. Hunt, F. Gottrup, G. C. Gurtner and M. T. Longaker, *Wound Repair Regen.*, 2009, **17**, 763-771.
2. T. Velnar, T. Bailey and V. Smrkolj, *The Journal of International Medical Research*, 2009, **37**, 1528-1542.
3. O. Bock, G. Schmid-Ott, P. Malewski and U. Mrowietz, *Arch. Dermatol. Res.*, 2006, **297**, 433-438.
4. C. G. Sheffield, 3rd, G. B. Irons, P. Mucha, Jr., J. F. Malec, D. M. Ilstrup and H. H. Stonnington, *J. Burn Care Rehabil.*, 1988, **9**, 172-177.
5. J. Li, J. Chen and R. Kirsner, *Clin. Dermatol.*, 2007, **25**, 9-18.
6. W. Liu, D. R. Wang and Y. L. Cao, *Curr. Gene Ther.*, 2004, **4**, 123-136.
7. M. Shah, D. M. Foreman and M. W. J. Ferguson, *J. Cell Sci.*, 1995, **108**, 985-1002.
8. A. B. Roberts, *Wound Repair Regen.*, 1995, **3**, 408-418.
9. S. J. Bates, E. Morrow, A. Y. Zhang, H. Pham, M. T. Longaker and J. Chang, *J. Bone Joint Surg. Am.*, 2006, **88**, 2465-2472.
10. C. S. Xia, X. Y. Yang, W. Yingzhen, K. Sun and S. Tian, *Orthopedics*, 2010, **33**, 727.
11. A double blind, placebo controlled trial to investigate the efficacy and safety of two concentrations of juvidex (Mannose-6-Phosphate) in accelerating the healing of split thickness skin graft donor sites using different dosing regimes, http://clinicaltrials.gov/ NCT00664352).
12. A. I. Arno, G. G. Gauglitz, J. P. Barret and M. G. Jeschke, *Burns*, 2014, **40**, 539-551.
13. S. Vidal, M. Garcia, J. L. Montero and A. Morere, *Bioorg. Med. Chem.*, 2002, **10**, 4051-4056.
14. E. C. Dell'Angelica and G. S. Payne, *Cell*, 2001, **106**, 395-398.
15. S. Hoogendoorn, G. H. M. van Puijvelde, J. Kuiper, G. A. van der Marel and H. S. Overkleeft, *Angew Chem Int Edit*, 2014, **53**, 10975-10978.
16. J. Zhang, M. G. Wong, M. Wong, W. Jarolimek, H. Schilter, J. Chen, C. Pollock and S. Saad, *Nephrology*, 2014, **19**, 56-57.
17. Y. T. Ko, A. Kale, W. C. Hartner, B. Papahadjopoulos-Sternberg and V. P. Torchilin, *J. Controlled Release*, 2009, **133**, 132-138.
18. T. Xia, M. Kovochich, M. Liong, H. Meng, S. Kabehie, S. George, J. I. Zink and A. E. Nel, *ACS Nano*, 2009, **3**, 3273-3286.
19. C. W. Evans, M. Fitzgerald, T. D. Clemons, M. J. House, B. S. Padman, J. A. Shaw, M. Saunders, A. R. Harvey, B. Zdyrko, I. Luzinov, G. A. Silva, S. A. Dunlop and K. S. Iyer, *ACS Nano*, 2011, **5**, 8640-8648.
20. J. Harrison, C. A. Bartlett, G. Cowin, P. K. Nicholls, C. W. Evans, T. D. Clemons, B. Zdyrko, I. A. Luzinov, A. R. Harvey, K. S. Iyer, S. A. Dunlop and M. Fitzgerald, *Small*, 2012, **8**, 1579-1589.
21. D. Fischer, T. Bieber, Y. Li, H. P. Elsasser and T. Kissel, *Pharm. Res.*, 1999, **16**, 1273-1279.
22. G. Malich, B. Markovic and C. Winder, *Toxicology*, 1997, **124**, 179-192.
23. M. R. Natowicz, M. M. Y. Chi, O. H. Lowry and W. S. Sly, *Proc. Natl. Acad. Sci. U. S. A.*, 1979, **76**, 4322-4326.
24. M. Gary-Bobo, P. Nirde, A. Jeanjean, A. Morere and M. Garcia, *Curr. Med. Chem.*, 2007, **14**, 2945-2953.
25. N. M. Dahms, L. J. Olson and J. J. P. Kim, *Glycobiology*, 2008, **18**, 664-678.
26. C. Z. C. Chen, Y. X. Peng, Z. B. Wang, P. V. Fish, J. L. Kaar, R. R. Koepsel, A. J. Russell, R. R. Lareu and M. Raghunath, *Br. J. Pharmacol.*, 2009, **158**, 1196-1209.
27. R. A. F. Clark, G. A. McCoy, J. M. Folkvord and J. M. McPherson, *J. Cell. Physiol.*, 1997, **170**, 69-80.
28. P. V. Fish, G. A. Allan, S. Bailey, J. Blagg, R. Butt, M. G. Collis, D. Greiling, K. James, J. Kendall, A. McElroy, D. McCleverty, C. Reed, R. Webster and G. A. Whitlock, *J. Med. Chem.*, 2007, **50**, 3442-3456.

TOC Graphic



Polymeric nanoparticle for delivery of an effective anti-fibrotic agent in an *in vitro* model of scarring